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14. ABSTRACT
The transforming growth factor (TGF) signaling pathway is an essential pathway whose initiation results in cell growth arrest in most epithelial cells. Activation of TGF receptors leads to the phosphorylation and translocation of the Smad proteins, the major TGF intracellular signaling molecule, to the nucleus where transcription of TGF target genes occur. Many breast cancers contain aberrations in the regulation of Smad proteins demonstrating the importance of TGF signaling. Therefore, understanding how post-translational modifications may regulate this pathway will increase our knowledge of how a normal cell becomes cancerous and may provide insight into novel therapeutics.

This proposal suggests a series of experiments designed to study the acetylation of Smad proteins. We have determined that Smad2 can be efficiently acetylated by the acetyltransferase protein p300 in vivo and in vitro and that this acetylation is necessary for the transcriptional activity of the protein in Smad2-deficient mouse embryonic fibroblasts. This decrease in transcriptional activity is due to the inability of non-acetylated Smad2 to accumulate in the nucleus upon TGF treatment and translates into marked decrease in the TGF induced cell cycle arrest that is essential for the tumor suppressing ability of the TGF signaling pathway.

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Introduction

The transforming growth factor-β (TGFβ) signaling pathway influences a diverse range of biological functions including cell growth arrest, differentiation, apoptosis, migration, and epithelial-to-mesenchymal transition (EMT) **(1-4).** Thus, mutations in the TGFβ pathway results in a myriad of human diseases such as prostrate and breast cancer **(5)**. TGFβ plays a complicated role cancer as it has both tumor suppressing and tumor promoting activities. The potent ability of TGFβ to initiate cell cycle arrest enables it function as a tumor suppressor in the early stages of tumorigenesis (5). However, in later stages of tumorigenesis TGFβ has been soon to initiate EMT and therefore increase the invasiveness and metastatic potential of the tumor, thus promoting tumorigenesis (6-7). Therefore, increasing our understanding of how the TGFβ signaling pathway is regulated will provide essential knowledge on how cancers arise and progress.

TGFβ signaling is initiated when TGFβ ligand bind to and activate the TGFβ receptor complex (8-9). The activated serine/threonine receptor complex then phosphorylates the c-terminal of Smad2 and Smad3, resulting in their oligomerization with the common mediator Smad4 (10-11). This Smad2/Smad3/Smad4 complex can then translocate to the nucleus where it recruits coactivators like p300/CBP to initiate transcription of TGFβ target genes (12-14).

The coactivators p300 and CBP function as scaffolding and bridging proteins as well as having intrinsic histone acetyltransferases abilities (15-17) p300/CBP directly interacts with the c-termini of Smad1, 2, 3, and 4 in a ligand dependent manner to increase the transcriptional activity of the Smad complex (13, 14, 18, 19). This interaction was originally thought to increase transcription by recruiting p300/CBP to the chromatin to modify it into its acetylated “open” conformation (20). However, p300/CBP has also demonstrated an ability to acetylate non-histone proteins such as p53 and Smad7 and alter a protein’s stability, binding ability, and localization (21-24.

This proposal seeks to elucidate the role Smad acetylation plays in regulating the TGFβ signaling pathway. Understanding the role of this modification in the TGFβ signaling pathway will suggest another possible mechanism that is likely to be perturbed in breast cancer, thus possibly leading to new ideas for the treatment or early detection of breast cancer.

Body

The TGFβ signaling pathway is initiated when TGFβ ligands bind to the constitutively active TGFβ type II serine/threonine receptor at the plasma membrane. This interaction results in the recruitment of the type I receptor that is then phosphorylated by the type II receptor, initiating a conformation change that activates the kinase domain of the type I receptor. The activated receptor complex can then recruit and phosphorylate the cytoplasmic proteins Smad2 and Smad3, enabling oligomerization with Smad4 and translocation of the entire Smad complex into the nucleus. Once in the nucleus, the activated Smad complex then recruits coactivators like the acetyltransferases p300/CBP and initiates transcription of TGFβ target genes.

Research performed prior to DOD funding determined that Smad2 but not Smad3 is efficiently acetylated in a p300 depend manner both in in vivo and in vitro models. This result is of particular interest because Smad2 and Smad3 have 93% sequence identity. Both proteins contain a conserved MH1 domain at the N-terminus linked to a conserved C-terminal MH2 domain by an unconserved proline-rich linker and both proteins are phosphorylated on a SSXS motif on the C-terminal tail of the protein. However, Smad2 contain two unique sequence inserts in the MH1 domain not found in Smad3 named the GAG and TID (also known as exon 3) region (25). These inserts provide steric interference that is responsible for the inability of Smad2 to directly bind DNA. Preliminary data also demonstrated that Smad2 acetylation was independent of Smad2 phosphorylation as Smad2 mutants where the SSXS motif has been mutated to AAXA were acetylated to the same extent as wild-type Smad2 in the presence of full-length p300.

In the approved statement of work, task one describes our plan to map the residues acetylated in Smad2. As stated in the annual report filed in 2005, we had originally planned on using Smad2 truncations to narrow down where the acetylation sites resided. However, the Smad2 truncations proved to be very unstable and therefore we were forced to modify our approach by using flag-tagged Smad1/Smad2 chimeras made by a previous graduate student identify the domain. Additional Smad1/Smad2 chimeras were cloned to further narrow down the acetylation site which was determined to reside upstream of the TID domain. Different combinations of lysines in the acetylation region were mutated to arginine and lysines 19 and 20 were found to be critical residues. To confirm lysine and 19 and 20 were the only residues necessary, mass spectrometry experiments were performed in 2006 and an additional lysine, lysine39, was found to also be modified. Thus, a non-acetylated mutant where lysines 19, 20, and 39 have been mutated to arginine (3K19R) and an acetylation mimicking mutant where lysines 19,20, and 39 were mutated to glutamine were cloned and used for biochemical studies.

We were also interested in learning what were the requirements necessary to see Smad2 acetylation. As stated in the original proposal, Smad2 acetylation appeared to occur even in a Smad2 mutant protein that could not be acetylated. Similarly, as stated in the 2005 annual report, the Smad2 3K19R

mutant was still efficiently phosphorylated demonstrating that phosphorylation and acetylation are mutually exclusive in an overexpression in vivo setting. Similarly, as reported in the 2005 annual report deletion studies proved the necessity of the GAG region for acetylation to occur as Smad2 mutants with the GAG region deleted lost all ability to be acetylated. Similarly, as reported in the 2006 annual report inserted a GAG region into Smad3 enabled the protein to be acetylated. Acetylation was also found to be a dynamic process as treatment with trichostatin A (TSA), a deacetylase inhibitor, increased the population of acetylated Smad2. Studies in 2007 found that acetylation of endogenous Smad2 requires stimulation with TGFβ and cellular fractionation studies were performed to determine where acetylated Smad2 protein exists. Purified cytoplasmic and nuclear fractions were western blotted for acetylated Smad2 protein and the modified protein was found to primarily exist in the nucleus upon treatment with TGFβ.

Task 2 described in the original proposal focused on characterizing the cellular consequences of Smad2 acetylation. We were interested in generated stable cell lines overexpressing the Smad2 3K19R mutant in Hep3B cells to how TGFβ responsive the cells would be. However, overexpressing the mutant Smad2 did not have any effect on the Hep3B cells. We hypothesized this lack in effect could occur because the endogenous Smad2 was masking any phenotype we would expect with the Smad2 3K19R mutants. Thus, we also attempted to Smad2 siRNA to knock-out Smad2 in Hep3B cells with the intention of adding back a Smad2 with a silent mutation in the siRNA complementary region. Unfortunately, the siRNA used did not significantly reduce the amount of Smad2 expressed in the Hep3B cells. Fortunately, Dr. Anita Robert's lab had previously generated Smad2-deficient mouse embryo fibroblasts that generously shared with us. Using this cells, we were able to generate a stable cell line expressing either wild type flag-tagged Smad2 or the flag-tagged Smad2 3K19R mutant. When treated with TGFβ, reintroducing wt Smad2 to the KO MEF cells rescued the cell's ability to undergo cell cycle arrest while reintroducing the Smad2 3K19R mutant protein did not. Thus, Smad2 acetylation is required for the tumor suppressor activity of the TGFβ signaling pathway.

Using the Smad2-deficient MEF cells obtained from the Roberts lab, we also tested the transcriptional activity of the wild type Smad2 and the Smad2 lysine to arginine/glutamine mutants. In both the case of activin and TGFβ signaling, adding back the wild type Smad2 and the Smad2 3K19Q mutant restored TGFβ- and activin-dependent transcription measured using luciferase reporter constructs. The lysine to arginine mutants were not able to restore the transcriptional activity measured supporting the observed lack of TGFβ induced cell cycle arrest in the stable cell lines.

What is the mechanism for this sudden loss in TGFβ responsiveness? Possible candidates included a change in protein stability, protein-protein interactions, protein-DNA binding, and protein subcellular localization. Using the Smad2 3K19R mutant we first used pulse chase experiments to determine whether acetylation could account for the high stability of the protein. After chasing the S35-radio-labelled protein for 0, 1, 4, 8, and 12 hours no discernable

difference could be detected between wild type Smad2 and the Smad2 3K19R mutant. Similarly co-immunoprecipitation experiments between flagged tagged Smad2 and the Smad2 3K19R mutant with myc tagged Smad4 demonstrated that both proteins were equally able to bind to Smad4. Gel shift assays also did not indicate a difference between the wild type and mutant protein when it came to DNA binding ability. Therefore, we turned our attention to the subcellular localization of wild type and the Smad2 mutant.

Using direct Immunofluorescence and confocal microscopy we were able to follow where wild type, Smad2 3K19R, and Smad3 3K19Q proteins localize upon stimulation with TGFβ. Studies have shown that in unstimulated cells Smad2 continually shuttles between the nucleus and the cytoplasm (26-28). The nuclear retention observed upon treatment with TGFβ is a direct consequence a decrease in the nuclear export rate (28). In multiple cell lines, treating cells transfected with wild type and the 3K19Q protein resulted in a strong nuclear accumulation not observed with the non-acetylated 3K19R mutant. To determine whether this lack of accumulation was due to a decrease in the export of the protein, cells transfected with wild type, 3K19R, and 3K19Q protein were treated with TGFβ and then treated with a TGFβ inhibitor SB-431542. In the case of the wild type protein, the protein accumulated in the nucleus prior to SB-431542 treatment and then quickly returned to the cytoplasm after treatment with the inhibitor. The 3K19R mutant never accumulated in the nucleus even upon TGFβ treatment as expected and therefore remained both nuclear and cytoplasmic during all time points taken. The 3K19Q mutant readily accumulated in the nucleus but did not redistribute to the cytoplasm as readily as the wild type protein after treatment with SB-431542. Therefore, acetylation of Smad2 contributes to the nuclear accumulation of Smad2 upon TGFβ stimulation by enhancing the nuclear retention of the protein.

The research described above provides insight into how acetylation of Smad2 can contribute to the suppression of tumorigenesis by enhancing the cell cycle arrest response in cell through the increased presence of the protein in the nucleus. Thus, acetylation is a vital modification necessary for the tumor suppressor abilities of the TGFβ signaling pathway. Continued research should examine whether acetylation of Smad2 also plays as crucial role in the tumorigenesis promoting capabilities of the protein to determine whether targeting the acetylation of Smad2 would be a feasible method of breast cancer treatment.

Key Research Accomplishments

- Identified residues modified through a combination of mutational analysis and mass spectrometry.
- Cloned non-acetylated and acetylation mimicking Smad2 mutants and made stable cell lines in Smad2-deficient MEF cells
- Worked out conditions to test characterize the effect of Smad2 acetylation on the TGFβ signaling pathway.

Reportable Outcomes – See Appendix

- Paper published
    - Tu AW, Luo K. (2007) "Acetylation of Smad2 by the co-activator p300 regulates activin and transforming growth factor beta response." *J Biol Chem.* **282**:21187-96.
- Data presented at the DOD Era of Hope 2004 conference in Philadelphia – June 2004

Conclusions

- Smad2 acetylation occurs on lysines 19, 20, and 39
- Smad2 acetylation requires the presence of the GAG domain.
- Endogenous Smad2 acetylation requires TGFβ stimulation.
- The Smad2 protein does not need to be phosphorylated to be acetylated
- Smad2 acetylation occurs in the nucleus.
- Smad2 acetylation is necessary for TGFβ- and activin-induced transcription.
- Smad2 acetylation is required for TGFβ induced cell cycle arrest response.
- Smad2 acetylation does no alter Smad2 phosphorylation, binding to Smad4, and DNA binding ability.
- Smad2 acetylation alters the subcellular localization of Smad2 upon TGFβ stimulation by decreasing the nuclear retention of the protein.

References

1. Massague J, BS, Lo RS, *TGFbeta signaling in growth control, cancer, and heritable disorders.* Cell, 2000. **103**(2): p. 295-309.

2. Massague J, aCY, *Controlling TGF-beta signaling.* Genes and Development, 2000. **14**: p. 627-644.

3. Whitman M, *Smads and early developmental signaling by the TGFbeta superfamily.* Genes and Development, 1998. **12**(16): p. 2445-62.

4. Zavadil, J. and E.P. Bottinger. *TGF-beta and epithelial-to-mesenchymal transitions*. Oncogene, 2005 **24**: 5764-74.

5. Blobe GS, SW, and Lodish HF, *Role of transforming growth factor beta in human disease.* N Engl J Med, 2000 **342:**1350-8.

6. Maehara Y et al, *Role of transforming growth factor-beta 1 in invasion and metastasis in gastric carcinoma. J Clin Oncol 17-607-14 (1999)*

7. Cui, W., D.J. Fowlis, S. Bryson, E. Duffie, H. Ireland, A. Balmain, and R.J. Akhurst. *TGFbeta1 inhibits the formation of benign skin tumors, but enhances progression to invasive spindle carcinomas in transgenic mice.* Cell,1996. **86**: 531-42.

8. Wrana J, AL, Carcamo J, Zentella A, Doody J, Laiho M, Wang X, and Massague J, *TGFbeta signals through a heteromeric protein kinase receptor complex.* Cell, 1992. **71**: p. 1003-1014.997.

9. Wrana J, AL, Wiser R, Ventura F, and Massague J, *Mechanism of activation of the TGFbeta receptor.* Nature,1994. **370**: p. 341-347.

10. Chen X, RM, and Whitman M, *A transcriptional partner for MAD proteins in TGFbeta signaling.* Nature,1996. **383**: p. 691-696.

11. Derynck R, ZY, and Feng XH, *Smads: transcriptional activators of TGFbeta responses.* Cell, 1998. **95**(6): p. 737-740.

12. Labbe E, ea, *Smad2 and Smad3 positively and negatively regulate TGF-beta dependent transcription through the forkhead DNA-binding protein.* Mol. Cell, 1998. **2**: p. 109-120.

13. Ayako Nishihara, J-iH, Nobuaki Okamoto, Jun Yanagisawa, Shigeaki Kato, Kohei Miyazono, and Masahira Kawabato, *Role of p300, a transcriptional coactivator, in signalling of TGF-*b. Genes to Cell, 1998. **3**: p. 613-623.

14. Ralf Janknecht, NJW, and Tony Hunter, *TGF*-b *stimulated cooperation of Smad proteins with the coactivators CBP/p300.* Genes and Development, 1998. **12**: p. 2114-2119.

15. Chrivia JC, KR, Lamb N, Hagiwara M, Montminy MR, Goodman RH., *Phosphorylated CREB binds specifically to the nuclear protein CBP.* Nature, 1993. **365**(6449): p. 855-9.

16. Arany Z, SW, Livingston DM, Eckner R., *E1A-associated p300 and CREB-associated CBP belong to a conserved family of coactivators.* Cell, 1994. **77**(6): p. 799-800.

17. Ogryzko VV, SR, Russanova V, Howard BH, Nakatani Y., *The transcriptional coactivators p300 and CBP are histone acetyltransferases.* Cell, 1996. **87**(5): p. 953-959.

18. Celio Pouponnot, LJ, and Joan Massague, *Physical and Functional Interaction of SMADS and p300/CBP.* The Journal of Biological Chemistry, 1998. **273**(36): p. 22865-22868.

19. Xin-Hua Feng, YZ, Rui-Yun Wu, and Rik Derynck, *The tumor suppressor Smad4/DPC4 and transcriptional coactivators for Smad3 in TGF*-b *induced transcriptional activation.* Genes and Development, 1998. **12**: p. 2153-2163.

20. Lusser, A, *Acetylated, methylated, remodeled: chromatin states for gene regulation.* Curr Opin Plant Biol, 2002. **5**(5): p. 437-443.

21. Gu W, RR, *Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain.* Cell, 1997. **90**(4): p. 595-606.

22. Gronroos E, HU, Heldin, CH, and Ericsson J, *Control of Smad7 Stability by Competition between Acetylation and Ubiquitination.* Molecular Cell, 2002. **10**: p. 483-493.

23. Soutoglou E, KN, and Talianidis I, *Acetylation Regulates Transcription Factor Activity at Multiple Levels.* Molecular Cell, 2000. **5**: p. 745-751.
24. Freiman RN, TR, *Regulating the regulators: lysine modifications make their mark.* Cell, 2003. **112**(1): p. 11-7.

25. Dennler S, HS, Gauthier J, *A short amino-acid sequence in MH1 domainis responsible for functional differences between Smad2 and Smad3.* Oncogene, 1999. **18**: p. 1643-1648.

26. Xu, L., Kang, Y., Col, S., and Massague, J. *Smad2 nucleocytoplasmic shuttling by nucleoporins CAN/Nup214 and Nup153 feeds TGFβ signaling complexes in the cytoplasm and nucleus*. Mol Cell, 2002 **10(2**), 271-282

27. Nicolas FJ, De Bosscher K, Schmierer B, Hill CS. *Analysis of Smad nuclecytoplasmic shuttling in living cells*. J Cell Sci , 2004 **117,** 4113-4125.

28. Schmierer, B., and Hill, C. S. *Kinetic analysis of Smad nucleopcytoplasmic shutting reveals a mechanism for transforming growth factor beta-dependent nuclear accumulation of Smads.* Mol Cell Biol*, 2005.* **25(22),** 9845-9858

Appendix I
Abstract from Era of Hope 2005 Conference

SMAD2 ACETYLATION IN THE TGF-β SIGNALING PATHWAY

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Cells have developed complex mechanisms to prevent inappropriate cell division from occurring. These precautions are important for the prevention of tumor growth and loss of these mechanisms can lead to tumor progression. One pathway used to control cell growth is the transforming growth factor-β (TGF-β) pathway. Initiation of the TGF-β signaling pathway results in a wide range of biological functions including cell growth arrest. Loss of regulation of the TGF-β signaling pathways has been observed in breast cancer and 50% of all pancreatic cancer. Therefore, understanding how TGF-β is regulated is essential in our understanding of how cancer arises and progresses.

The TGF-β signaling pathway is mediated primarily through the intracellular Smad proteins. Many cancer cells have displayed either decreased levels or mutations in the Smad proteins, indicating an important role in the prevention of cancer progression. Smad proteins are regulated at several stages of TGF-β signaling through protein modifications such as phosphorylation and interactions with other proteins.

Recently, the receptor regulated Smad2 was identified as an acetylated protein through in vitro acetylation assays. Acetylation, a relatively new post-translations modification, has been shown to affect protein stability, localization, and DNA and protein binding ability. The acetylation of Smad2 was further confirmed by co-transfecting different Smad proteins in the presence of p300 into cells and performing a Western Blot using an antibody specific to acetylated lysines (Fig 1). Interestingly, Smad3, another receptor regulated Smad with 92% sequence identity to Smad2, cannot be acetylated.

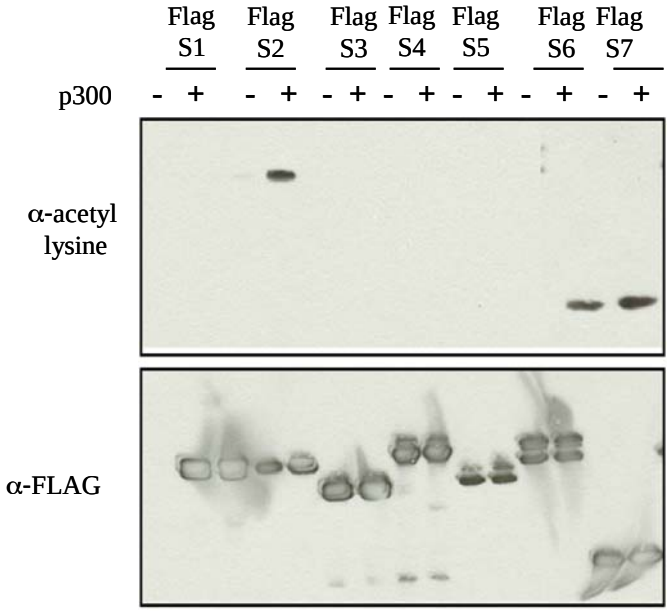

Fig1: Fl-Smad proteins transfected into 293T cells. Cells were lysed and FLAG proteins immunoprecipitated. The product was run on SDS-PAGE gel and subjected to Western blots with the indicated antibodies

A series of Smad1/Smad2 chimeras indicated that acetylation of Smad2 occurs in the MH1 domain of Smad2. Swapping

the MH1 domain of Smad2 and Smad3 confirms that acetylation occurs in the MH1 domain. Point mutations of lysines in the MH1 domain identified lysines 19 and 20 as residues required for Smad2 acetylation (Fig. 2). Performing mass spec on acetylated Smad2 will test whether lysines 19/20 are the actual sites of acetylation or if they are merely regulatory.

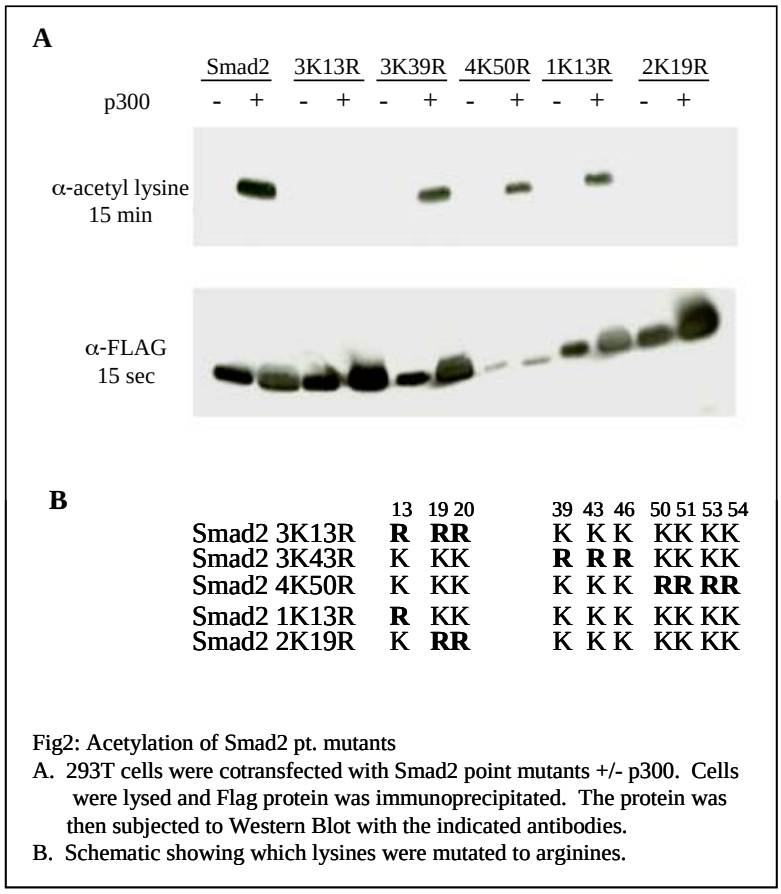

Fig2: Acetylation of Smad2 pt. mutants
A. 293T cells were cotransfected with Smad2 point mutants +/- p300. Cells were lysed and Flag protein was immunoprecipitated. The protein was then subjected to Western Blot with the indicated antibodies.
B. Schematic showing which lysines were mutated to arginines.

Functional tests to determine the consequences of Smad2 acetylation will be conclusively performed. Preliminary results suggest that a loss of Smad2 acetylation does not play a role in the protein's stability nor does it alter its phosphorylation state. Other potential factors affected by acetylation to be checked include protein-protein interactions, protein-DNA interactions, and localization of the protein. Ascertaining the role of Smad2 acetylation in the TGF-β pathway gives us further insight as to how abrogations in the TGF-β pathway can lead to initiation and progression of breast cancer. It may also lend insight into how other pathways implicated in breast cancer are regulated. All this information only increases our knowledge of breast cancer and will ultimately lead to better forms of prevention, detection, and treatment of this disease.

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Appendix II

Paper published:

- Tu AW, Luo K. (2007) “Acetylation of Smad2 by the co-activator p300 regulates activin and transforming growth factor beta response.” *J Biol Chem.* **282**:21187-96.

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Acetylation of Smad2 by the Co-activator p300 Regulates Activin and Transforming Growth Factor β Response*

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Transforming growth factor β (TGFβ) signals primarily through the Smad proteins to regulate cell growth, differentiation, and extracellular matrix production. Post-translational modifications, such as phosphorylation, play an important role in the regulation of the Smad proteins. TGFβ signaling results in the phosphorylation of Smad2 and Smad3 that then oligomerize with Smad4 and translocate into the nucleus to initiate transcription of TGFβ target genes. The initiation of transcription is significantly enhanced by the direct interaction of the Smad complex with p300/CBP (CREB-binding protein), a co-activator with intrinsic acetyltransferase activity. However, how p300/CBP enhances transcription through this interaction is not entirely understood. In this report, we show that Smad2, but not the highly homologous Smad3, can be acetylated by p300/CBP in a ligand-dependent manner. At least three lysine residues, Lys[19], Lys[20], and Lys[39], are required for efficient acetylation of Smad2, as mutations altering these lysines abolished Smad2 acetylation *in vivo*. This acetylation event is required for the ability of Smad2 to mediate activin and TGFβ signaling. Mutation of the three key lysine residues did not alter the stability of Smad2 or the ability of Smad2 to form a complex with Smad4 on promoter DNA, but it prevented nuclear accumulation of Smad2 and subsequent TGFβ and activin responses. Thus, our studies reveal a novel mechanism of modulating Smad2 activity and localization through protein acetylation.

The transforming growth factor β (TGFβ)[2] signaling pathway plays complex roles in the regulation of many diverse biological processes including cell cycle arrest, differentiation, apoptosis, and epithelial to mesenchymal transition. TGFβ signaling is initiated when ligands of the TGFβ signaling superfamily such as activin and TGFβ bind to and activate the TGFβ serine/threonine kinase receptor complex (1, 2). The activated receptor complex then phosphorylates receptor-activated Smads, such as Smad2 and Smad3, on the SS*X*S motif in the C termini, enabling nuclear translocation and oligomerization with the common mediator Smad4 (3, 4). In the nucleus, this Smad heteromeric complex binds to target promoter sequences and recruits transcriptional coactivators to regulate transcription of TGFβ target genes (1, 5).

Smad2 and Smad3 mediate both activin and TGFβ signaling and share 92% sequence identity. However, there are several salient differences between the two. First of all, Smad2 contains two extra peptide inserts, named the GAG and TID regions, respectively, that are not present in Smad3 (6). The TID domain (also known as exon3) in Smad2 prevents the direct binding of Smad2 to DNA, whereas Smad3 can bind DNA directly (7). Secondly, mice lacking Smad2 or Smad3 display very different phenotypes. Smad2 knock-out mice are embryonic lethal at embryonic day 10.5 (E10.5) with vascular and cranial abnormalities and impaired left-right patterning (8–10). Smad3-null mice, however, are viable but suffer from impaired immune function and chronic inflammation (11, 12). Finally, the stability and intracellular localization of Smad3 and Smad2 are also regulated by different mechanisms (13). Thus, many functional and regulatory differences exist between these two proteins.

Both Smad2 and Smad3 bind to and recruit the coactivator p300/CBP to enhance transcriptional activity of the activated Smad complex. p300 was first identified as an E1A-associated protein and displays high levels of sequence and functional homology with the CREB-binding protein, CBP (14). Initially identified as a scaffolding protein to bridge two proteins together, p300 also functions to transfer the acetyl group from acetyl coenzyme A to the lysine residues in histones allowing remodeling of chromatin to a more open relaxed conformation for transcription (15–17). Acetylation of histones can be reversed by the activity of histone deacetylases (HDAC), which returns the active chromatin back to its closed, inactive form by removal of acetyl groups (18).

In the past decade, many non-histone proteins, such as p53, β-catenin, importin-α, E1A, and Smad7 (19–21), have been shown to be acetylated by p300 and other acetyltransferase proteins such as P/CAF and GCN. Acetylation of non-histone proteins can result in alterations of biochemical and functional activities of the substrate proteins. For example, acetylation appears to inhibit the interaction of E1A with importin-α, decreasing its nuclear import (22). Acetylation can alter the intracellular localization of proteins such as c-Abl and can also compete with other modifications such as ubiquitination and

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[2] The abbreviations used are: TGFβ, transforming growth factor β; CBP, CREB-binding protein; CREB, cAMP-response element-binding protein; HDAC, histone deacetylase; TSA, trichostatin A; FBS, fetal bovine serum; HA, hemagglutinin; MEF, mouse embryo fibroblast; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; WT, wild type; EMSA, electrophoretic mobility shift assay.

sumoylation in proteins like p53 and Smad7 (21, 23, 24). Acetylation of Smad7, an inhibitory Smad, increases its stability by modifying the lysine residues required for ubiquitin attachment, preventing ubiquitination and proteasomal degradation (25). Finally, acetylation may alter the intracellular localization, DNA binding, and other post-translational modifications of proteins (26).

The MH2 domains of Smad1, Smad2, Smad3, and Smad4 interact directly with the C-terminal domain of p300/CBP, resulting in increases in the transcriptional activity of the Smad complex (27–31). Disruption of this interaction through overexpression of adenovirus E1A significantly decreases transcription by the Smad complex (32). Smad3 has also been shown to interact with HDAC1 directly through its MH1 domain and recruit it to the promoter DNA to inhibit transcription (33). Based on these observations, it has been proposed that the Smad proteins activate transcription by recruiting the p300/CBP histone acetyltransferase to the chromatin to remodel it into the active, open confirmation (34). However, whether this is the only mechanism by which p300/CBP enhances Smad transcription is not clear. Indeed, Inoue *et al.* (35) recently reported acetylation of Smad3 on lysine 378 by p300/CBP in cells treated with trichostatin A (TSA). While this manuscripts was being prepared, a new study by Simonsson *et al.* (36) also reported identification of an acetylation site in Smad2 that appears to be important for the DNA binding activity of the alternatively spliced form of Smad2 but has no effect on the signaling activity of WT Smad2. In this study, we carried out a detailed analysis of acetylation of Smad proteins by p300. We show that Smad2, but not Smad3, can be acetylated in a p300-dependent manner and that this modification requires lysines 19, 20, and 39 in Smad2. We further show that this acetylation event plays a role in promoting the nuclear accumulation of Smad2 upon TGFβ stimulation, leading to the increase in downstream TGFβ responses. Our work thus has uncovered a novel mechanism by which p300/CBP enhances TGFβ signaling through direct modification of Smad2.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—293T and NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium containing 10% FBS. Hep3B human heptoma cells (American Type Culture Collection) were maintained in minimum Eagle's medium supplemented with 10% FBS. Smad2-deficient mouse embryonic fibroblasts obtained from Dr. Anita Roberts were cultured in Dulbecco's modified Eagle's medium containing 10% FBS, sodium pyruvate (1 mM), and glutamate (2 mM).

Monoclonal antibodies specific for acetylated lysine were purchased from Cell Signaling Technologies. Smad2 antibodies were from BD Transduction Laboratories and Smad3 (FL-425) and TGFβ receptor (TGFβRI; V22) antibodies from Santa Cruz Biotechnology. Anti-FLAG, anti-hemagglutinin (HA) antibodies and TSA were purchased from Sigma. Anti-phospho-Smad2 antisera were a generous gift from Aristidis Moustakas (Ludwig Institute for Cancer Research, Uppsala, Sweden). SB-431542 was purchased from Tocris.

Transfection and Stable Cell Lines—Transient transfections were performed using Lipofectamine Plus reagents (Invitrogen) according to manufacturer's protocol. To generate stable cell lines, Smad2-null mouse embryo fibroblast (MEF) cells were co-transfected with wild type or mutant FLAG-Smad2 and pBABEpuro, which contains a puromycin resistance gene. After 48 h, transfected cells were selected by growing in medium containing 2 μg/ml puromycin.

Immunoprecipitation and Immunoblotting—Cells were lysed in high salt lysis buffer (50 mM Hepes, pH 7.8, 500 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 3 mM DTT, and 0.5 mM PMSF). FLAG-Smad2 was isolated by immunoprecipitation with anti-FLAG followed by elution with FLAG peptide as described previously (37, 38). Western blotting was carried out as described previously (39).

In Vitro Acetylation Assay—Purified GST-Smad fusion proteins were incubated at 30 °C for 30 min in acetylation assay buffer (50 mM Tris, pH 7.8, 1 mM DTT, 10% glycerol, 1 mM PMSF, 10 mM sodium butyrate, 1 mM EDTA, and 0.05 μCi of ^{14}C-acetyl coenzyme A (PerkinElmer Life Sciences) in the presence of GST-tagged histone acetylation domain of p300 (GST-p300-HAT). Samples were resolved on a SDS-polyacrylamide gel and visualized by autoradiography using a phosphorimaging system.

Mass Spectrometry—FLAG-tagged Smad2, isolated from 293T cells co-transfected with full-length p300 by immunoprecipitation with anti-FLAG, were eluted with FLAG peptide and resolved on a 10% SDS-polyacrylamide gel. The Smad2 band was then excised from the gel, digested with chymotrypsin, and run through a Thermo Finnigan LCQ DECA XP Plus ion trap mass spectrometer interfaced with a Shimadzu Binary HPLC to carry out liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

Luciferase Assay—MEF cells were transiently transfected with the promoter reporter constructs and FLAG-tagged wild type or mutant Smad2. At 24 h after transfection, cells were serum-starved and treated with 50 pM TGFβ for 16 h. Luciferin levels were measured as described previously (38).

Growth Inhibition Assay—1×10^4 MEF cells were seeded in 6-well plates and stimulated with different concentrations of TGFβ1 for 4 days. Cells were counted and compared with the number of unstimulated cells to determine relative cell growth.

Pulse-Chase Assay—293T cells were co-transfected with full-length p300 and FLAG-tagged wild type or mutant Smad2. At 24 h after transfection, cells were pulsed with 0.5 μCi/ml [^{35}S]methionine (PerkinElmer Life Sciences) in pulse media (cysteine/methionine-free Dulbecco's modified Eagle's medium plus 10% dialyzed FBS) for 30 min. Cells were then washed with regular media and chased for different periods of time. ^{35}S-Labeled Smad2 was then isolated by immunoprecipitation, resolved on a 10% SDS-polyacrylamide gel, and detected by autoradiography.

Electrophoretic Mobility Shift Assay (EMSA)—DNA fragments containing the Smad-binding element as described previously (40) were end-labeled with ^{32}P, gel-purified, and incubated with affinity-purified Smad2-Smad4 complex to test for DNA binding ability. The protein-DNA complexes were resolved on a 5% nondenaturing gel. For antibody supershift assays, the Smad2-Smad4 complexes were preincubated with 4

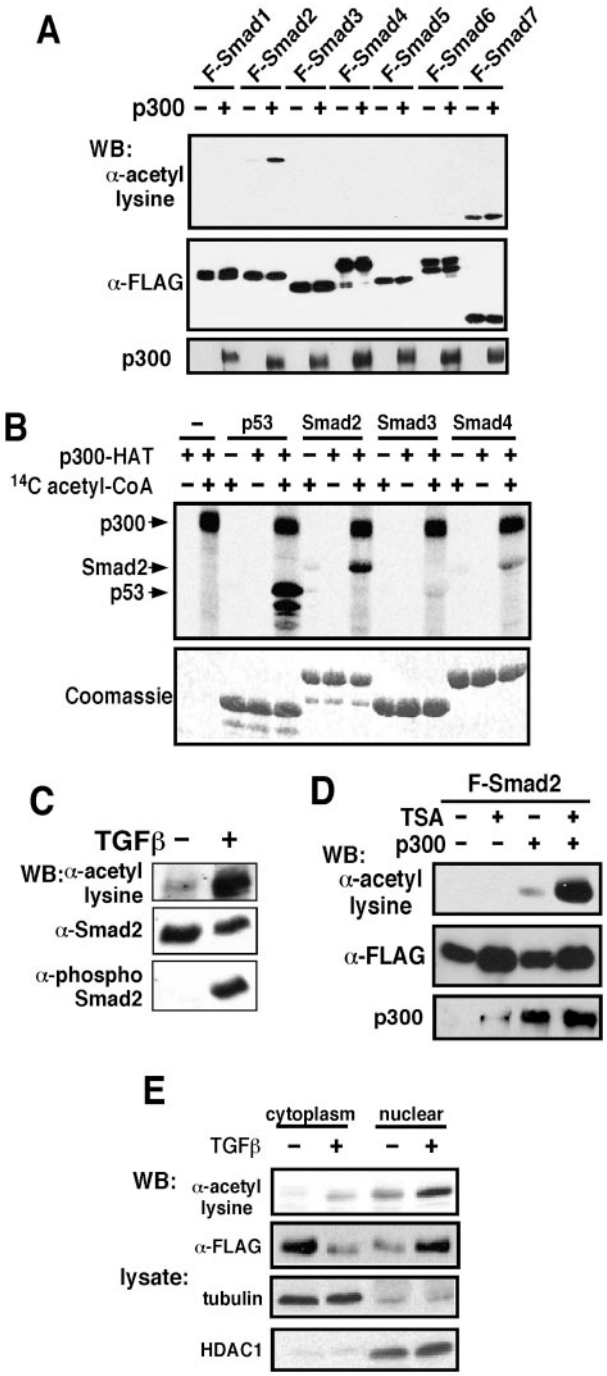

FIGURE 1. **Smad2 is acetylated by p300.** *A*, FLAG-tagged Smads (*F-Smad*) were transiently transfected into 293T cells in the absence and presence of full-length p300 and isolated by immunoprecipitation as described under "Experimental Procedures." Acetylation of these Smad proteins was detected by Western blotting (*WB*) with an acetyl lysine-specific antibody. The membrane was stripped and reprobed using an anti-FLAG antibody. *B*, *in vitro* acetylation assay. Bacterially expressed GST-p53 and GST-Smad proteins were incubated with the acetyltransferase domain of p300 and ^{14}C-labeled acetyl-CoA. The ^{14}C-labeled proteins were resolved on an SDS-polyacrylamide gel, dried, and detected by phosphorimaging. *C*, Hep3B cells were serum-starved and treated with 2.5 μM TSA for 8 h. Cells were then stimulated with 100 pM TGFβ and harvested. Endogenous Smad2 was isolated by immunoprecipitation with anti-Smad2 followed by Western blotting with the indicated antibodies. *D*, Hep3B cells were transiently transfected with FLAG-Smad2 (*F-Smad2*) in the absence and presence of full-length p300. 24 h after transfection, cells were treated with 1 μM TSA for 12 h. Smad2 was isolated by immunoprecipitation with anti-FLAG followed by Western blotting with the indicated antibodies. *E*, Hep3B cells were transiently transfected with FLAG-Smad2 and treated with 1 μM TSA for 12 h. Cells were stimulated with 100 pM TGFβ, cytoplasmic and nuclear fractions were isolated, and lysates were probed for tubulin and HDAC1 as a control. Smad2 was isolated by immunoprecipitation with anti-FLAG followed by Western blotting with an anti-acetyl lysine antibody.

μg of the specified antibody for 1 h at 4 °C before EMSA was performed.

Immunofluorescence—Cells were seeded on sterile glass slides and treated with 100 ng/ml TSA for 8 h before staining. Cells were fixed with 4% paraformaldehyde, permeablized with 0.1% Triton X-100, blocked with blocking buffer (10% newborn calf serum, 1% bovine serum albumin, and 0.02% Triton X-100 in phosphate-buffered saline), and stained with anti-FLAG as described previously (41). Proteins were visualized on an Axiophot epifluorescence microscope or a confocal LSM 510 microscope (Zeiss).

To study protein shuttling, cells were treated for 30 min with 100 μg/ml cycloheximide before stimulation with 100 pM TGFβ. Cells were then treated with 10 μM SB-431542 prior to immunostaining.

Cell Fractionation Assay—Cells were fractionated as described previously (42). Briefly, cells were lysed using Buffer A (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 0.5 mM PMSF) to isolate the cytoplasmic fraction. The remaining lysate was then resuspended in Buffer C (20 mM Hepes, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF) to extract the nuclear contents.

RESULTS

Smad2 Is Acetylated in the Presence of p300—To determine whether Smad proteins could be acetylated, a panel of FLAG-tagged Smad proteins were transiently transfected into 293T cells together with full-length p300 (Fig. 1*A*). Smad7 had been shown previously to be acetylated in the presence of p300 (21) and therefore was included as a positive control. Smad proteins were isolated by immunoprecipitation with anti-FLAG beads, and their acetylation status was evaluated by Western blotting with anti-acetyl lysine-specific antiserum. Under this condition, in addition to Smad7, only Smad2 was acetylated when coexpressed with p300. Interestingly, the highly homologous Smad3 was not significantly acetylated. The observed difference in acetylation between Smad2 and Smad3 is particularly interesting because of the high sequence identity between the two.

To determine whether p300 could directly acetylate Smad2, an *in vitro* acetylation assay was performed using bacterially expressed Smad2, Smad3, and Smad4 and the purified acetyltransferase domain of p300 (Fig. 1*B*) or CBP (data not shown). Again, strong acetylation of Smad2 was observed in the presence of p300 or CBP, suggesting that p300/CBP can directly acetylate Smad2. In the same reaction, Smad3 and Smad4 also appeared to be labeled, but these signals were much weaker if significant at all when compared with that of Smad2. Thus, we decided to focus on the acetylation of Smad2 for the rest of the study.

Endogenous Smad2 Is Also Acetylated—To demonstrate that endogenous Smad2 is also acetylated and that this acetylation event is subjected to regulation by TGFβ, endogenous Smad2

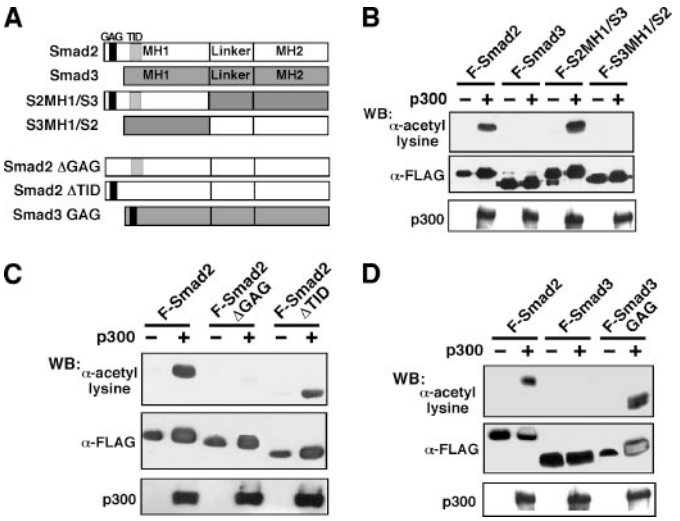

FIGURE 2. **GAG domain is necessary and sufficient for Smad2 acetylation.** *A*, Smad2 mutations. *B–D*, FLAG-tagged Smad2 constructs were transiently transfected into 293T cells in the absence and presence of full-length p300 and isolated by immunoprecipitation with anti-FLAG. Acetylation of Smad2 was analyzed by Western blotting (*WB*).

was isolated from Hep3B cells treated with or without TGFβ by immunoprecipitation with anti-Smad2 and subjected to Western blotting with anti-acetyl lysine-specific antibody. In the absence of TGFβ, Smad2 is acetylated but at a very low level. Treatment with TGFβ induced increased acetylation of Smad2 (Fig. 1*C*). This was expected because p300 is localized only in the nucleus, and its interaction with Smad2 increases upon nuclear accumulation of Smad2 as a result of TGFβ stimulation. This acetylation of Smad2 was further enhanced by treatment with TSA, an inhibitor of HDACs, suggesting that Smad2 acetylation is reversible and can be regulated by HDACs (Fig. 1*D*).

To further confirm that acetylation of Smad2 indeed occurs in the nucleus, cytoplasmic and nuclear fractions were prepared from Hep3B cells, and acetylation of Smad2 was determined as described above. A low level of acetylated Smad2 could be detected in the nuclear fraction in the absence of TGFβ stimulation, but this acetylation was significantly enhanced in the presence of TGFβ (Fig. 1*E*). In contrast, no acetylated Smad2 was detected in the cytoplasmic fractions either in the presence of absence of TGFβ. Thus, acetylation of Smad2 occurs primarily in the nucleus.

The GAG Region of Smad2 Is Required for Acetylation—To identify the sites of acetylation in Smad2, we co-transfected Smad2/Smad3 chimeras swapping the MH1 or MH2 domains of Smad2 together with p300 into 293T cells and evaluated the acetylation of these mutants by Western blotting (Fig. 2, *A* and *B*). The N-terminal MH1 domain appeared to be necessary and sufficient for Smad2 acetylation (Fig. 2*B*). Within the MH1 domain, the major difference between Smad2 and Smad3 is the presence of the GAG and TID domains in Smad2 but not in Smad3. Deletion of the GAG domain from Smad2 significantly impaired Smad2 acetylation, whereas removal of the TID domain had no effect (Fig. 2*C*), suggesting that acetylation of Smad2 requires the GAG region. Consistent with this, when the GAG region was inserted into Smad3, acetylation of Smad3 was observed at levels comparable with that of wild type Smad2 (Fig. 2*D*). Thus, the GAG domain of Smad2 is both necessary and sufficient for Smad2 acetylation.

Identifying Smad2 Acetylation Sites—There are many lysine residues in the MH1 domain of Smad2, most of which are conserved in Smad3 with the exception of Lys^{144}, which is uniquely present in Smad2. However, mutation of this lysine did not affect the acetylation of Smad2 (data not shown), indicating that Lys^{144} is not the site of acetylation. It is likely, therefore, that the GAG region of Smad2 confers a difference in conformation between Smad2 and Smad3 that causes the surrounding lysines in Smad2 to be more accessible to acetylation by p300.

Two complementary approaches were taken to identify the sites of acetylation in Smad2. In the first approach, we employed mass spectrometry to detect lysine residues with *N*-linked acetylation modifications in FLAG-Smad2 isolated from 293T cells co-overexpressing p300. Mass spectrometry identified three lysine residues, lysines 19, 20, and 39, as possible acetylated residues (Fig. 3*B*). In the second approach, point mutations changing combinations of lysines to arginines (Fig. 3*A*) in the area proximal to the GAG region were generated and examined for acetylation. Mutation of lysines 19 and 20, either by themselves or in combination with other lysine residues (for example 3K13R; see Fig. 3*A* for diagram of Smad2 Lys-Arg point mutants), resulted in a significant loss of Smad2 acetylation, whereas mutations of all other lysine residues in this area did not affect acetylation (Fig. 3*C*). This suggests that Lys^{19} and Lys^{20} are required for Smad2 acetylation. However, they are not the only sites of acetylation, because the 2K19R mutant was still acetylated effectively in an *in vitro* acetylation assay (Fig. 3*D*). This is consistent with the result obtained from the mass spectrometry analysis showing that Lys^{39} is also an acetylation site. Although mutation of Lys^{39} alone did not significantly affect Smad2 acetylation and only partially inhibited its transcriptional activity (see Fig. 5), mutation of Lys^{19}, Lys^{20}, and Lys^{39} abolished acetylation and Smad2 signaling (Figs. 3*C* and 5). Taken together, our study suggests that Smad2 can be acetylated on three lysine residues, Lys^{19}, Lys^{20}, and Lys^{39}.

Smad2 Phosphorylation and Acetylation Occur Independently of Each Other—Stimulation of TGFβ results in the phosphorylation of Smad2 in the C-terminal serine residues, leading to its oligomerization with Smad4 and nuclear translocation. To elucidate whether the C-terminal phosphorylation of Smad2 is necessary for acetylation, a Smad2 3S-3A mutant lacking the C-terminal phosphorylation sites was transfected into 293T cells along with full-length p300 (Fig. 4*A*). Although endogenous Smad2 needs to be phosphorylated to accumulate in the nucleus, immunofluorescence staining confirmed that overexpressed Smad2 3S-3A can bypass this requirement and accumulate in the nucleus even without TGFβ stimulation (Fig. 4*B*). When thus overexpressed, the Smad2 3S-3A mutant was acetylated to a similar level as the wild type protein, indicating that Smad2 in the nucleus does not need to be phosphorylated for acetylation to occur.

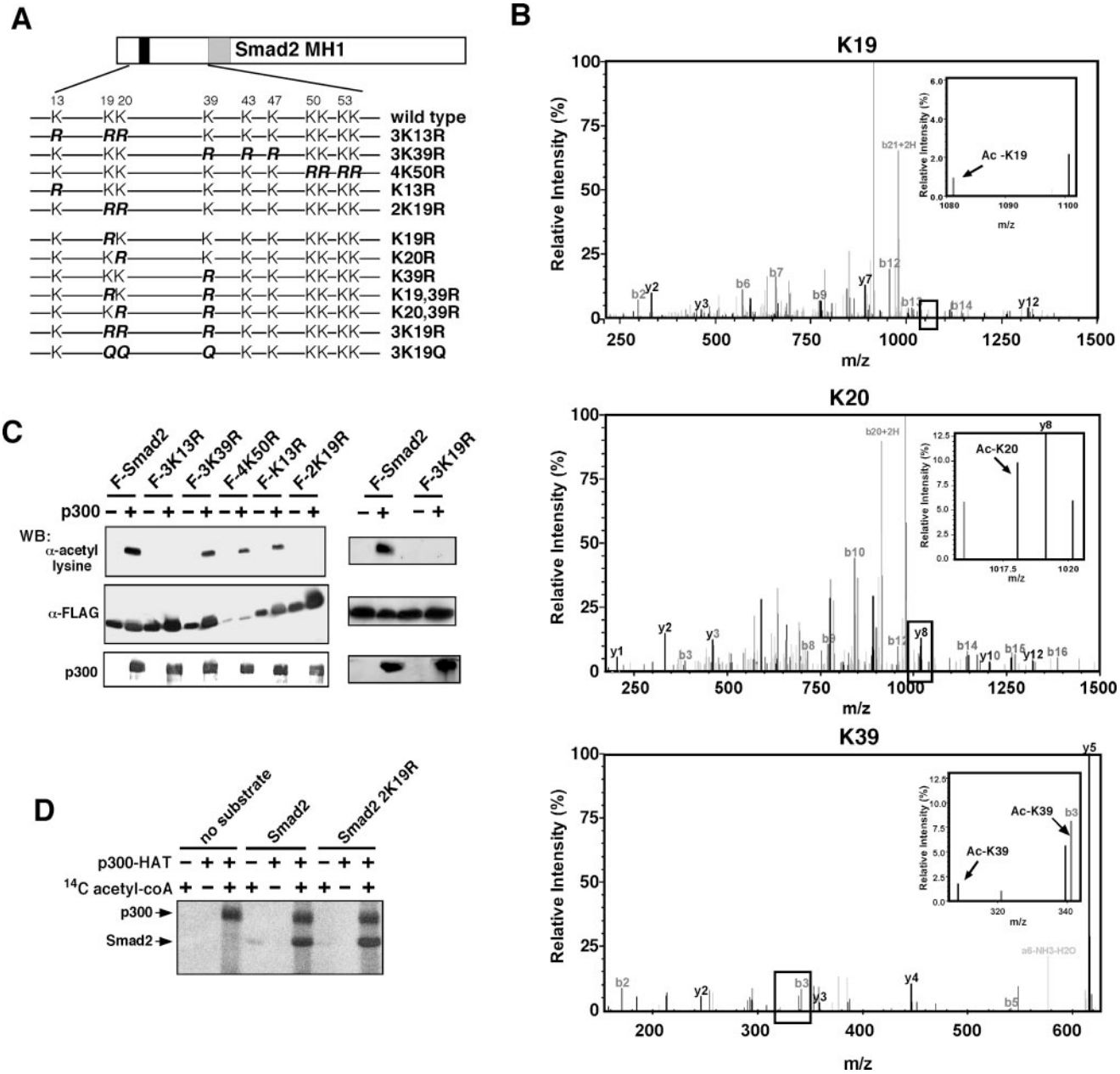

FIGURE 3. **Lysines 19, 20, and 39 are acetylated residues.** *A*, Smad2 point mutations. *B*, mass spectrometry analysis. Acetylated F-Smad2 was separated on 10% SDS-PAGE, and the Smad2 band digested in-gel with chymotrypsin for mass spectrometry analysis. Data containing N-terminal b ions (*black*), C-terminal y ions (*gray*), and unmatched ions (*light gray*) were analyzed using the programs Mascot and Scaffold. Peaks signifying lysines with increased mass were tagged (*insets*) and labeled based on the peptide placement in the fragment. *C*, FLAG-tagged Smad2 point mutants were transiently transfected into 293T cell in the absence and presence of full-length p300 and isolated by immunoprecipitation with anti-FLAG. Acetylation of Smad2 was analyzed by Western blotting (*WB*). *D*, *in vitro* acetylation assay. Bacterially expressed GST-Smad2 proteins were incubated with the acetyltransferase domain of p300 and ^{14}C-labeled acetyl-CoA as described under "Experimental Procedures."

Similarly, acetylation of Smad2 did not alter the phosphorylation state of the protein. Wild type and 3K19R mutant Smad2 were phosphorylated to similar levels when co-transfected with p300 into 293T cells (Fig. 4*C*). Thus, although phosphorylation of Smad2 is necessary to for its nuclear translocation, once in the nucleus acetylation of Smad2 can occur independently of its phosphorylation status.

Acetylation of Smad2 Is Required for Activin and TGFβ Signaling—To determine the effects of Smad2 acetylation on TGFβ signaling, we first measured TGFβ- and activin-dependent transcription using luciferase reporter constructs specific for activin (ARE-lux) or TGFβ (p3TP-lux) signaling. Western blotting was performed to ensure equal levels of Smad2 and 3K19R expression in the transfected cells (data not shown). MEFs isolated from the Smad2-null mice (43) were used to avoid any potential interference by endogenous Smad2.

Unlike WT MEF, which displayed strong activation of ARE-lux upon activation of activin signaling, Smad2-null MEF no longer supported transcription from ARE-lux (Fig. 5*A*). Transfection of wild type Smad2 restored the transcription activation to the Smad2-deficient MEF. In contrast, introduction of the 3K19R mutant did not rescue activin-induced transcriptional

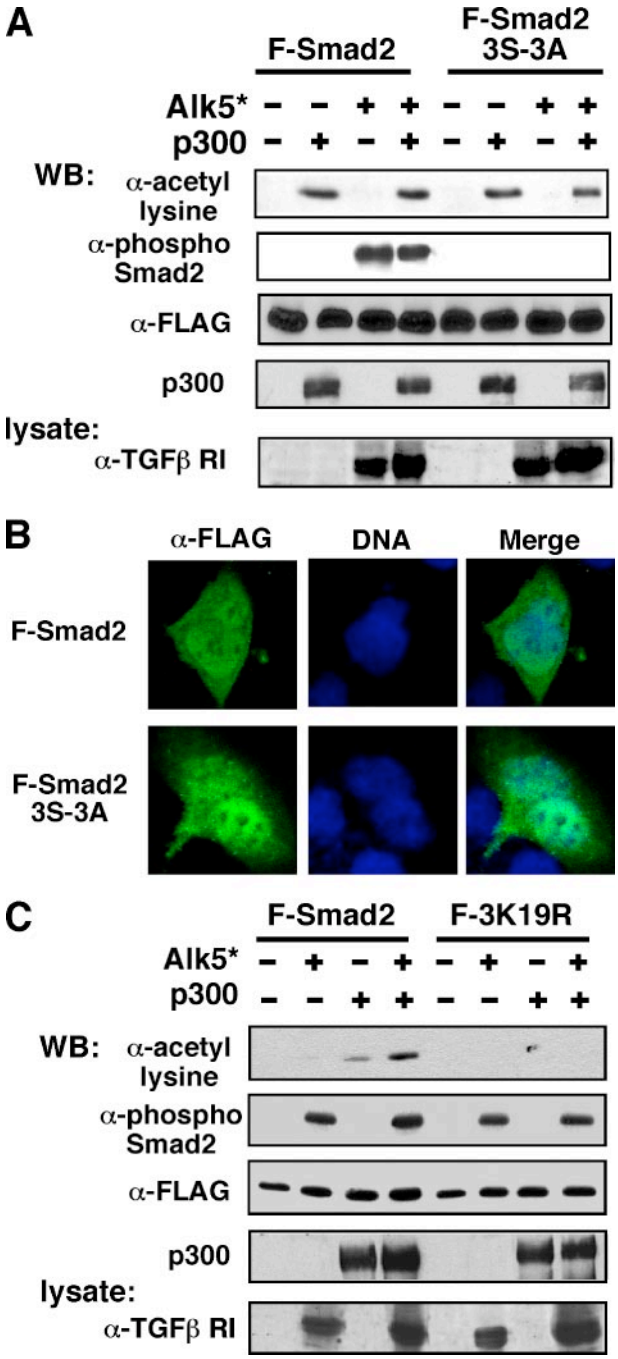

FIGURE 4. **Phosphorylation and acetylation of Smad2 are independent of each other.** *A*, FLAG-tagged wild type and 3S-3A mutant Smad2 were transiently transfected into 293T cells in the presence and absence of full-length p300. Acetylation of Smad2 was analyzed by Western blotting (*WB*). *B*, FLAG-tagged Smad constructs were transiently transfected into Hep3B cells. Wild type F-Smad2 (*F-Smad2*) and F-Smad2 3S-3A were localized through immunostaining with anti-FLAG and visualized using confocal microscopy. *C*, wild type and 2K19R mutant Smad2 were transfected into 292T cell in the presence and absence of full-length p300 and constitutively active TGFβ type I receptor (Alk5*). Phosphorylation of Smad2 was assessed by Western blotting with anti-phospho-Smad2 antibodies.

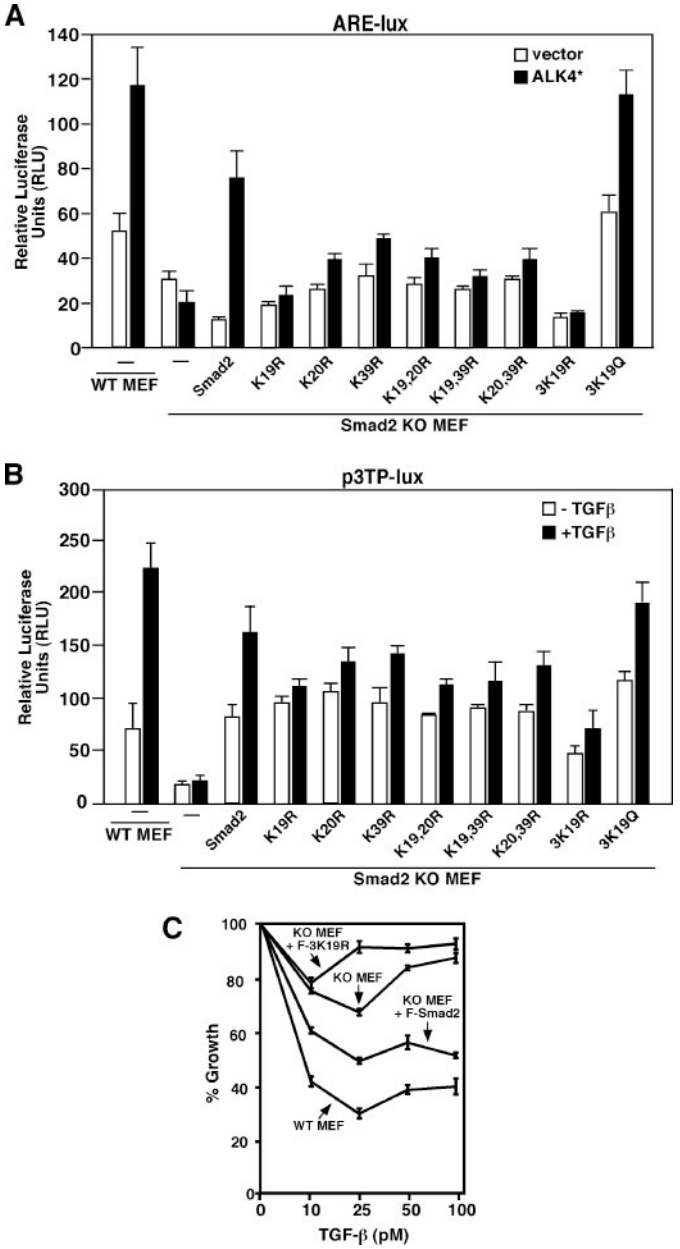

FIGURE 5. **Acetylation of Smad2 is required for activin and TGFβ signaling.** WT or mutant Smad2 was co-transfected into Smad2-null MEF together with either ARE-lux (*A*) or p3TP-lux (*B*). Cells were treated with 50 pM TGFβ for16 h, and a luciferase assay was performed as described under "Experimental Procedures." *C*, WT or 3K19R Smad2 was stably introduced into Smad2-null MEF. Cells were treated with increasing concentration of TGFβ for 4 days, and cell growth was evaluated by cell counting.

activation. Similarly, wild type but not the 3K19R mutant conferred TGFβ-induced transactivation to Smad2-null MEF (Fig. 5*B*). Thus, acetylation of Smad2 is required for activin- and TGFβ-induced transcriptional responses. Consistent with this, a mutant that mimics the acetylated state of Smad2 (3K19Q) exhibited a moderate but reproducible increase in transcription (Fig. 5, *A* and *B*) when expressed at a similar level as WT Smad2 (data not shown).

To discern the contribution of each of the three acetylated lysine residues to activin- and TGFβ-induced transcription responses, mutations altering each of the individual lysine residues (K19R, K20R, K39R) or two in combination (K19R/K20R, K19R/K39R, K20R/K39R) (Fig. 3*A*) were generated and tested in the luciferase reporter assay. All of these mutants partially inhibited the transcriptional activity of Smad2, suggesting that acetylation of all three lysine residues contribute to the activity

of Smad2. The Lys[19] mutants demonstrated the most significant decrease in transcriptional activation, most likely because this residue is necessary for the cooperative acetylation of other lysines. Thus, mutating this lysine also affects the acetylation potential of the other identified acetylated lysines (Fig. 5, *A* and *B*).

We next examined how Smad2 acetylation affects the growth inhibitory response of TGFβ. Wild type or the 3K19R mutant Smad2 was transfected stably into the Smad2-null MEF. Cells were treated with different concentrations of TGFβ, and the growth of cells was assessed after 4 days (Fig. 5*C*). WT MEFs expressing endogenous Smad2 were responsive to TGFβ, exhibiting ~60% growth inhibition, whereas Smad2-null MEF showed very little growth inhibition. Stable expression of wild type Smad2 suppressed cell growth ~50% whereas cells expressing the 3K19R mutant did not exhibit any cell cycle arrest. Taken together, these results indicate that acetylation of Smad2 is required for activin and TGFβ signaling.

Acetylation Does Not Affect the Stability, Smad4 Oligomerization, and DNA Binding Ability of Smad2—We next turned to the molecular mechanism by which acetylation of Smad2 affects its signaling activity. In particular, we examined the effects of Smad2 acetylation on its stability, the ability to hetero-oligomerize with Smad4, its presence in the DNA-binding complex, and intracellular localization. In pulse-chase experiments using transfected 293T cells, both the wild type Smad2 and the 3K19R mutant have similar half-lives (Fig. 6*A*), suggesting that Smad2 stability is not affected by acetylation.

Because Smad2 must oligomerize with Smad4 to carry out its transcription activity, we next examined whether acetylation affected its interaction with Smad4 by a co-immunoprecipitation assay (Fig. 6*B*). In 293T cells transfected with FLAG-tagged wild type or mutant Smad2 together with Myc-Smad4 and the constitutively active TGFβ receptor (Alk5*), wild type and 3K19R mutant associated with similar amounts of Smad4, indicating that acetylation does not alter the oligomerization of Smad2 with Smad4.

Similarly, in an EMSA using the Smad2-Smad4 complex purified from transiently transfected 293T cells, no difference in DNA binding ability was detected between the wild type Smad2 and the 2K19R mutant, suggesting that acetylation of Smad2 does not affect DNA binding (Fig. 6*C*).

Acetylation of Smad2 Affects Intracellular Localization after TGFβ Treatment—Acetylation has been shown to play a role in the intracellular localization of many proteins including p53, HNF-α, and importin-α. To elucidate whether Smad2 acetylation also influences its intracellular localization, we performed indirect immunofluorescence staining in Hep3B cells transfected with wild type or mutant Smad2 (Fig. 7). In the absence of TGFβ, both wild type and mutant Smad2 were distributed throughout the cell in both cytoplasm and nucleus, consistent with reports that under nonstimulated conditions Smad2 quickly shuttles in and out of the nucleus (44). Upon TGFβ treatment, wild type Smad2 rapidly accumulated in the nucleus as expected. In contrast, the 3K19R mutant remained in the cytoplasm and did not accumulate in the nucleus. Similar results were also observed in NIH3T3 cells and Smad2-null MEF cells (data not shown). Single lysine to arginine and double

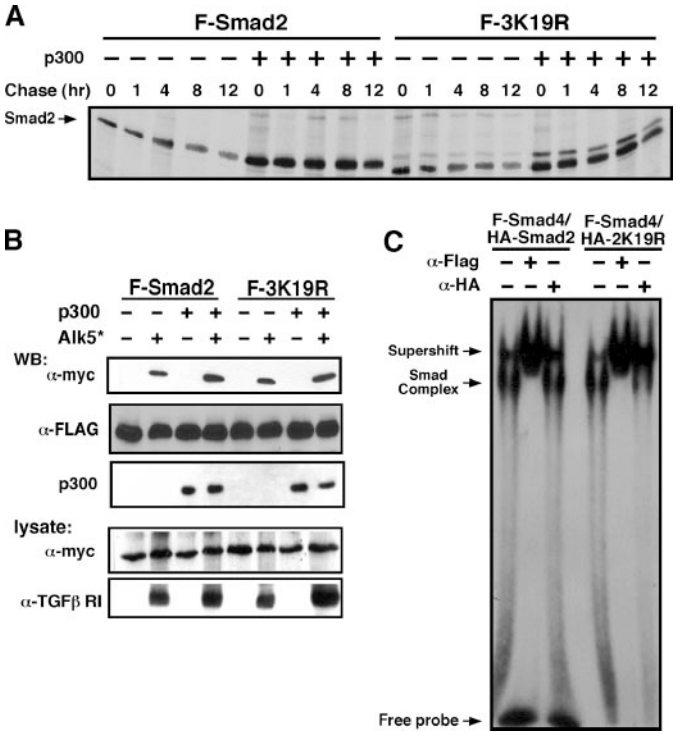

FIGURE 6. **Smad2 acetylation does not affect Smad2 stability, oligomerization with Smad4, or its presence on promoter DNA.** *A*, pulse-chase assay. 293T cells were transfected with wild type or 3K19R Smad2 in the presence and absence of full-length p300, pulse-labeled, and chased. ^{35}S-Labeled Smad2 were isolated and resolved by SDS-PAGE as described under "Experimental Procedures." *B*, wild type or 3K19R Smad2 was co-transfected into 293T cells together with Myc-tagged Smad4 in the absence and presence of full-length p300 and the constitutively active TGFβ type I receptor (*RI*), Alk5*. Smad2 was isolated by immunoprecipitation with anti-FLAG, and the associated Smad4 was detected by Western blotting (*WB*) with anti-Myc antibody. *C*, EMSA. The Smad2-Smad4 complex was isolated from 293T cells transfected with FLAG-Smad4, a constitutively active TGFβ type I receptor (Alk5*), and HA-tagged wild type or 2K19R Smad2. An EMSA and a supershift assay were performed as described under "Experimental Procedures."

lysine to arginine mutants also exhibited a failure to fully accumulate in the nucleus, whereas the 3K19Q mutant, which mimics the acetylation state of Smad2, localized readily in the nucleus upon TGFβ stimulation (Fig. 7). Thus, acetylation of Smad2 appears to be required for its nuclear translocation in response to TGFβ. This decreased nuclear accumulation in response to TGFβ most likely accounts for the inability of the mutant Smad2 to mediate TGFβ and activin signaling.

Acetylation May Affect Nuclear Export—If acetylation of Smad2 increases its nuclear accumulation, one would predict that the acetylation-mimicking 3K19Q mutant will linger in the nucleus longer following TGFβ stimulation. To test this, we performed a time course experiment examining the localization of WT Smad2 and 3K19Q at various times after TGFβ stimulation. Hep3B cells transiently expressing WT or mutant Smad2 were pretreated with cycloheximide prior to TGFβ treatment to prevent new protein synthesis. After 1 h of TGFβ treatment, the ALK5 receptor inhibitor SB-431542 was added to the cells to block further activation of the Smad proteins. Localization of Smad2 proteins at various time points after these treatments was determined by immunofluorescence staining. By this approach we were able to follow the fate of activated Smad2.

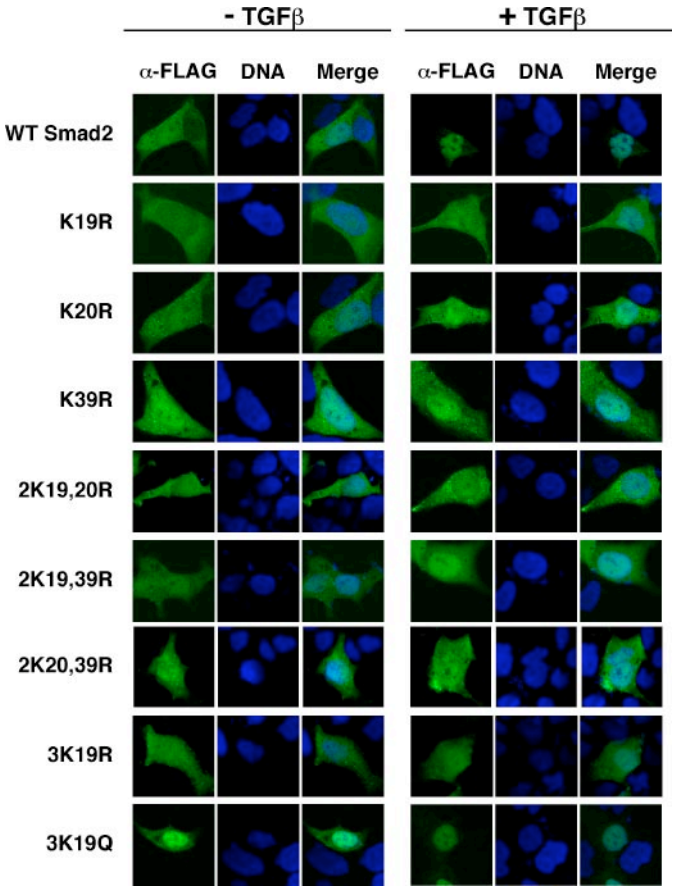

FIGURE 7. **Smad2 acetylation is required for nuclear accumulation.** Hep3B cells were transfected with 0.1 μg of WT or 3K19R-Smad2 in the absence and presence of full-length p300. Cells were treated with TSA for 10 h and with 100 pM TGFβ for 1 h prior to staining. Smad2 localization was determined by immunostaining with anti-FLAG and visualized using confocal microscopy.

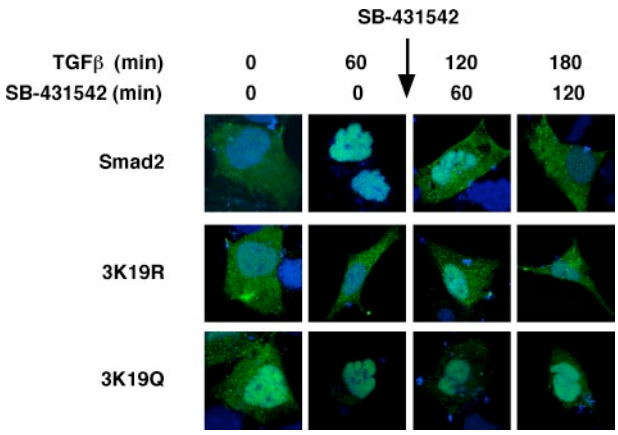

FIGURE 8. **Smad2 acetylation may decrease the rate of nuclear export.** Hep3B cells transiently expressing WT or mutant Smad2 in the presence of full-length p300 were pretreated with 100 μg/ml cycloheximide for 30 min before treatment with 100 pM TGFβ. Cells were treated either with or without SB-451542 for the indicated times prior to immunostaining with anti-FLAG and visualization using confocal microscopy.

Consistent with previously published results (45), WT Smad2 accumulated in the nucleus at 1 h after TGFβ stimulation but relocated back to the cytoplasm at 2 h post-stimulation. In contrast, the 3K19Q mutant readily accumulated in the nucleus in response to TGFβ but failed to be redistributed back to the cytoplasm even after 3 h of TGFβ treatment (Fig. 8). This observation confirms that acetylation promotes nuclear localization of Smad2 and further suggests that it may do so by decreasing nuclear export of Smad2.

DISCUSSION

Reversible protein acetylation has been shown to affect a diverse array of biochemical properties including protein-protein interactions, DNA binding, protein stability, and intracellular localization. In this study we have demonstrated that Smad2, but not Smad3, can be robustly acetylated in the presence of p300/CBP both *in vitro* and *in vivo* to enhance TGFβ and activin signaling. This acetylation requires at least three key lysine residues, lysines 19, 20, and 39, and appears to promote Smad2 nuclear accumulation, leading to enhanced transcription activity. We have shown that acetylation of Smad2 appears to affect the localization of Smad2 upon TGFβ signaling possibly by decreasing the rate of Smad2 nuclear export following TGFβ stimulation. The 3K19R mutant that could no longer be acetylated failed to accumulate in the nucleus, whereas the 3K19Q mutant that mimicked a constitutively acetylated state remained in the nucleus much longer than WT Smad2 after TGFβ signaling ends.

Smad2 has been shown to continuously shuttle in and out of the nucleus (45), and TGFβ-induced nuclear accumulation occurs primarily as a result of a decrease in the rate of nuclear export (46). Our result that acetylation affects nuclear export is entirely consistent with these earlier observations and also to be expected because import of Smad2 precedes acetylation by nuclear p300. It is not clear how acetylation directly influences nuclear export. There are at least two possibilities. First, acetylation may affect the dephosphorylation of Smad2, which is necessary for relocation of Smad2 to the cytoplasm (47). Alternatively, acetylation may directly impact the export machinery. Xu *et al.* (48) have shown that Smad2 directly binds to the nucleoporins CAN/Nup214 and Nup153 and that Nup153 is necessary for Smad2 export in an importin/exportin-independent manner. Whether this mechanism is the only one responsible for Smad2 export *in vivo* is not clear. Much more work is clearly needed in the future to determine the exact mechanism by which acetylation prevents nuclear export. Nevertheless, our data suggest that in conjunction with Smad phosphorylation, Smad2 acetylation contributes to the slowing of nuclear export following TGFβ stimulation and therefore constitutes an important step in the regulation of TGFβ and activin signaling.

Smad2 is not the only protein in the TGFβ signaling pathway that can be acetylated. In addition to Smad7, Inoue *et al.* (35) recently reported acetylation of Smad2 and Smad3 by p300/CBP in cells treated with TSA. They found that acetylation of Smad3 occurred in the MH2 domain on Lys^{378}, a lysine conserved among all R-Smads. Mutation of this lysine to arginine (K378R) appeared to result in a decrease in receptor-mediated phosphorylation of Smad3 and subsequently a reduction in transcriptional activity. However, we did not observe very robust acetylation of Smad3, especially compared with that of Smad2, nor did mass spectrometry experiments identify Lys^{378} as an acetylated residue under our

experimental conditions. While this manuscripts was being prepared, a new report by Simonsson *et al.* (36) also found acetylation of Smad3 to occur at a significantly lower level than that of Smad2. In contrast to our observation that mutant Smad2 defective in acetylation exhibited impaired signaling activity, they detected no reduction in transcriptional activity of the K19R mutant Smad2 in HepG2 cells using the ARE-lux reporter assay. We speculate that the reason that they failed to observe a difference between wild type and mutant Smad2 in transcription may be because of the interference of endogenous Smad2 in HepG2 cells, as we did not observe any difference in transcription activity between wild type and mutant Smad2 in Hep3B cells either (Data not shown). The endogenous Smad2 in these cells could be functioning at saturation levels, masking the effect of the mutant Smad2. When we used Smad2-null MEF, a clear difference could be readily observed.

Smad2 exists in two isoforms: the full-length protein form containing both the GAG and TID (exon3) domains and an alternatively spliced short form lacking exon 3 (Smad2ΔE3) (49). Unlike full-length Smad2, this shorter isoform is able to bind DNA directly. Simonsson *et al.* (36) showed that acetylation on Lys^{19} in the short isoform Smad2(ΔE3) is required for its DNA binding activity. However, because the full-length Smad2 does not bind to DNA directly, acetylation is unlikely to have the same effect on full-length Smad2. Indeed, we showed that the 3K19R mutant Smad2 can still be recruited to DNA through oligomerization with Smad4. Instead, acetylation of full-length Smad2 enhances Smad2 activity by promoting its nuclear accumulation through decreasing the rate of nuclear export. Because the full-length Smad2 is found in all adult and embryonic tissues but Smad2(ΔE3) is present primarily in mouse cells during development, acetylation may regulate the activity of these Smad2 forms through different mechanisms in embryos and adult tissue.

Like phosphorylation, acetylation can occur on multiple lysine residues within the same protein and often can occur in different combinations depending on the specific conditions of the cells and the given acetyltransferase enzymes, leading to various downstream consequences. Although lysines 19, 20, and 39 were found to be required for Smad2 acetylation, they may not be the only residues that are acetylated. Additional Smad2 acetylation sites may exist that may conceivably affect Smad2 activity. Identification of specific acetylation sites may be difficult, as the specificity of acetyltransferase for substrate lysine residues is not stringent so that, in the absence of the targeted lysine, other lysine residues in the proximity may serve as substrates. Despite this difficulty, a continuing effort to decipher Smad2 acetylation sites under different stimuli and conditions will surely increase our understanding of how the TGFβ signaling pathway is regulated by acetylation. Our findings also provide a possible new direction for pharmaceutical intervention of TGFβ signaling in cancer and inflammatory diseases by targeting Smad2 acetylation. Treatment of cells with HDAC inhibitors may increase the nuclear accumulation of Smad2, leading to improved growth-inhibitory responses of cells response to TGFβ.

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REFERENCES

1. Feng, X. H., and Derynck, R. (2005) *Annu. Rev. Cell Dev. Biol.* **21,** 659–693
2. Miyazono, K., ten Dijke, P., and Heldin, C. H. (2000) *Adv. Immunol.* **75,** 115–157
3. Abdollah, S., Macias-Silva, M., Tsukazaki, T., Hayashi, H., Attisano, L., and Wrana, J. L. (1997) *J. Biol. Chem.* **272,** 27678–27685
4. Miyazono, K. (2000) *Cytokine Growth Factor Rev.* **11,** 15–22
5. ten Dijke, P. H. C. (2004) *Trends Biochem. Sci.* **29,** 265–273
6. Dennler, S., Huet, S., and Gauthier, J. M. (1999) *Oncogene* **18,** 1643–1648
7. Shi, Y., Wang, Y. F., Jayaraman, L., Yang, H., Massague, J., and Pavletich, N. P. (1998) *Cell* **94,** 585–594
8. Heyer, J., Escalante-Alcalde, D., Lia, M., Boettinger, E., Edelmann, W., Stewart, C. L., and Kucherlapati, R. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 12595–12600
9. Nomura, M., and Li, E. (1998) *Nature* **393,** 786–790
10. Waldrip, W. R., Bikoff, E. K., Hoodless, P. A., Wrana, J. L., and Robertson, E. J. (1998) *Cell* **92,** 797–808
11. Datto, M. B., Frederick, J. P., Pan, L., Borton, A. J., Zhuang, Y., and Wang, X. F. (1999) *Mol. Cell Biol.* **19,** 2495–2504
12. Yang, X., Letterio, J. J., Lechleider, R. J., Chen, L., Hayman, R., Gu, H., Roberts, A. B., and Deng, C. (1999) *EMBO J.* **18,** 1280–1291
13. Massague, J., Seoane, J., and Wotton, D. (2005) *Genes Dev.* **19,** 2783–2810
14. Janknecht, R. (2002) *Histol. Histopathol.* **17,** 657–668
15. Chan, H. M., and La Thangue, N. B. (2001) *J. Cell Sci.* **114,** 2363–2373
16. Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H., and Nakatani, Y. (1996) *Cell* **87,** 953–959
17. Tsukiyama, T., and Wu, C. (1997) *Curr. Opin. Genet. Dev.* **7,** 182–191
18. Wu, J., and Grunstein, M. (2000) *Trends Biochem. Sci.* **25,** 619–623
19. Gu, W., and Roeder, R. G. (1997) *Cell* **90,** 595–606
20. Wolf, D., Rodova, M., Miska, E. A., Calvet, J. P., and Kouzarides, T. (2002) *J. Biol. Chem.* **277,** 25562–25567
21. Gronroos, E., Hellman, U., Heldin, C. H., and Ericsson, J. (2002) *Mol. Cell* **10,** 483–493
22. Madison, D. L., Yaciuk, P., Kwok, R. P., and Lundblad, J. R. (2002) *J. Biol. Chem.* **277,** 38755–38763
23. Li, M., Luo, J., Brooks, C. L., and Gu, W. (2002) *J. Biol. Chem.* **277,** 50607–50611
24. Melchior, F., and Hengst, L. (2002) *Cell Cycle* **1,** 245–249
25. Simonsson, M., Heldin, C. H., Ericsson, J., and Gronroos, E. (2005) *J. Biol. Chem.* **280,** 21797–21803
26. Glozak, M. A., Sengupta, N., Zhang, X., and Seto, E. (2005) *Gene* **363,** 15–23
27. Feng, X. H., Zhang, Y., Wu, R. Y., and Derynck, R. (1998) *Genes Dev.* **12,** 2153–2163
28. Janknecht, R., Wells, N. J., and Hunter, T. (1998) *Genes Dev.* **12,** 2114–2119
29. Nishihara, A., Hanai, J. I., Okamoto, N., Yanagisawa, J., Kato, S., Miyazono, K., and Kawabata, M. (1998) *Genes Cells* **3,** 613–623
30. Pouponnot, C., Jayaraman, L., and Massague, J. (1998) *J. Biol. Chem.* **273,** 22865–22868
31. Shen, X., Hu, P. P., Liberati, N. T., Datto, M. B., Frederick, J. P., and Wang, X. F. (1998) *Mol. Biol. Cell* **9,** 3309–3319
32. Nishihara, A., Hanai, J., Imamura, T., Miyazono, K., and Kawabata, M. (1999) *J. Biol. Chem.* **274,** 28716–28723
33. Liberati, N. T., Moniwa, M., Borton, A. J., Davie, J. R., and Wang, X. F. (2001) *J. Biol. Chem.* **276,** 22595–22603

34. Ross, S., Cheung, E., Petrakis, T. G., Howell, M., Kraus, W. L., and Hill, C. S. (2006) *EMBO J.* **25,** 4490–4502
35. Inoue, Y., Itoh, Y., Abe, K., Okamoto, T., Daitoku, H., Fukamizu, A., Onozaki, K., and Hayashi, H. (2007) *Oncogene* **26,** 500–508
36. Simonsson, M., Kanduri, M., Gronroos, E., Heldin, C. H., and Ericsson, J. (2006) *J. Biol. Chem.* **281,** 39870–39880
37. Zhou, Q., Chen, D., Pierstorff, E., and Luo, K. (1998) *EMBO J.* **17,** 3681–3691
38. Stroschein, S. L., Wang, W., Zhou, S., Zhou, Q., and Luo, K. (1999) *Science* **286,** 771–774
39. Luo, K., Stroschein, S. L., Wang, W., Chen, D., Martens, E., Zhou, S., and Zhou, Q. (1999) *Genes Dev.* **13,** 2196–2206
40. Zhu, Q., Pearson-White, S., and Luo, K. (2005) *Mol. Cell Biol.* **25,** 10731–10744
41. Krakowski, A. R., Laboureau, J., Mauviel, A., Bissell, M. J., and Luo, K. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 12437–12442
42. Lee, K. A., Bindereif, A., and Green, M. R. (1988) *Gene Anal. Tech.* **5,** 22–31
43. Piek, E., Ju, W. J., Heyer, J., Escalante-Alcalde, D., Stewart, C. L., Weinstein, M., Deng, C., Kucherlapati, R., Bottinger, E. P., and Roberts, A. B. (2001) *J. Biol. Chem.* **276,** 19945–19953
44. Pierreux, C. E., Nicolas, F. J., and Hill, C. S. (2000) *Mol. Cell. Biol.* **20,** 9041–9054
45. Inman, G. J., Nicolas, F. J., and Hill, C. S. (2002) *Mol. Cell* **10,** 283–294
46. Schmierer, B., and Hill, C. S. (2005) *Mol. Cell. Biol.* **25,** 9845–9858
47. Lin, X., Duan, X., Liang, Y. Y., Su, Y., Wrighton, K. H., Long, J., Hu, M., Davis, C. M., Wang, J., Brunicardi, F. C., Shi, Y., Chen, Y. G., Meng, A., and Feng, X. H. (2006) *Cell* **125,** 915–928
48. Xu, L., Kang, Y., Col, S., and Massague, J. (2002) *Mol. Cell* **10,** 271–282
49. Dunn, N. R., Koonce, C. H., Anderson, D. C., Islam, A., Bikoff, E. K., and Robertson, E. J. (2005) *Genes Dev.* **19,** 152–163